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Quantitation of Microsomal α-Hydroxylation of the Tobacco-specific Nitrosamine, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone[1]

Lisa A. Peterson,[2] Rachel Mathew, and Stephen S. Hecht

Division of Chemical Carcinogenesis, American Health Foundation, Valhalla, New York 10595

ABSTRACT

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is activated to DNA alkylating species via two different α-hydroxylation pathways. Methylene hydroxylation leads to DNA methylation, whereas methyl hydroxylation yields DNA pyridyloxobutylation. We have developed a high-pressure liquid chromatography assay utilizing radiochemical detection that permits the determination of the extent of metabolism through each pathway in microsomal preparations. Levels of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) were used to measure the extent of methyl hydroxylation, whereas levels of the aldehyde, 4-oxo-1-(3-pyridyl)-1-butanone (OPB), were used to quantify the extent of methylene hydroxylation. Incubations of [5-^{3}H]NNK with microsomes and cofactors were conducted in the presence of 5 mM sodium bisulfite to trap the reactive OPB. The inclusion of bisulfite did not affect the rate of NNK metabolism. Trapping the aldehyde also inhibited its further oxidation to the corresponding acid or reduction to HPB. Furthermore, the conversion of HPB to OPB made only a minor contribution to the OPB levels under our incubation conditions. Analysis of incubation mixtures containing [5-^{3}H]NNK, cofactors, and either A/J mouse liver or lung microsomes demonstrated that OPB was a significant metabolite of NNK. The OPB:HPB ratio was greater in liver (1.5) than in lung (0.2–1) microsomal preparations. Apparent K_m values for OPB and HPB formation in lung microsomes were 23.7 and 3.6 μM, respectively, whereas the corresponding values for liver microsomes were 19.1 and 73.8 μM, respectively. These data are consistent with the involvement of more than one cytochrome P-450 isozyme in the activation of NNK to DNA reactive species.

INTRODUCTION

NNK,[3] a tobacco-specific nitrosamine, is a potent lung carcinogen in laboratory animals (1–3). NNK requires metabolic activation to elicit its carcinogenic effects (1). It is an asymmetric nitrosamine that can be activated through two different α-hydroxylation pathways (Fig. 1). Methyl hydroxylation leads to formation of 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone which decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide and formaldehyde. This diazohydroxide can react with water to generate HPB or it can react with DNA to form pyridyloxobutyl adducts. On the other hand, methylene hydroxylation produces 4-hydroxy-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone which decomposes to methanediazohydroxide and OPB. Methanediazohydroxide can either react with DNA to form methylated bases or react with water to yield methanol.

Both pathways are involved in the *in vivo* metabolism of NNK since both pyridyloxobutyl and methyl DNA adducts are detected in tissues from NNK-treated rats and mice (4–7). The extent of NNK metabolized through each pathway is not known. These two pathways cannot be distinguished *in vivo* because both HPB and OPB are further oxidized to OPBA. Furthermore, relative amounts of DNA pyridyloxobutylation or methylation do not shed light on this question since the two diazohydroxides have different rates of DNA reactivity.[4] Since the pyridyloxobutylation and methylation routes produce adducts with differing biological effects in mice (6), it is important to quantify relative rates of oxidation at the two α-carbon atoms.

The measurable primary metabolic products of the two pathways are HPB and formaldehyde for methyl hydroxylation and OPB and methanol for methylene hydroxylation. The availability of [5-^{3}H]NNK, which has tritium at position 5 of the pyridine ring, allows for detection of HPB and OPB in small quantities in the absence of isotope effects. [5-^{3}H]HPB can be detected without derivatization. OPB, on the other hand, is reactive and has not been observed directly in microsomal incubations (8). This metabolite has been detected as its 2,4-dinitrophenylhydrazone derivative but only at high NNK concentrations (9). The potential for further oxidation of OPB to OPBA, as well as its reactive dicarbonyl structure, can confound attempts to quantitate this metabolite. Therefore, it is desirable to conduct incubations in the presence of a trapping agent that could compete with protein nucleophiles for reaction with OPB, but that does not interfere with the metabolism rate of NNK. Chemical derivatization of the aldehyde should also prevent its further oxidation to OPBA.

This paper describes the development of an assay that quantitates the two α-hydroxylation pathways of NNK. Bisulfite was an efficient trapping agent for OPB when coincubated with [5-^{3}H]NNK and microsomal preparations. Therefore, we were able to determine the extent of methylene hydroxylation by measuring the amount of OPB-bisulfite adduct and the extent of methyl hydroxylation by determining the levels of HPB. We have used this assay to determine these activities in A/J mouse liver and lung microsomal preparations.

MATERIALS AND METHODS

Chemicals. NNK, HPB, [5-^{3}H]HPB, and OPB were synthesized as previously described (7, 9–11). [5-^{3}H]NNK (2.1 Ci/mmol) was purchased from ChemSyn (Lenexa, KS) and purified by C_{18} HPLC chromatography by using HPLC method C (see below). Sodium bisulfite, bovine serum albumin, NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO).

HPLC Chromatographic Systems. Three different chromatographic systems were used in these studies. Method A utilized solvent A (20 mM sodium phosphate buffer, pH 7) and solvent B (95% methanol, 5% water), using a gradient from 100% solvent A to 70% solvent A over 60 min (12). The flow rate was 1 ml/min. OPB-bisulfite adduct, OPB, and HPB coeluted under these conditions at 52 min on a C_{18} column (B&J ODS, 5 μm, 25 × 0.46 cm).

Method B elutes the metabolites off the same column with solvent C (20 mM sodium acetate buffer, pH 4.5) and solvent B. The following

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; OPB, 4-oxo-1-(3-pyridyl)-1-butanone; HPLC, high-pressure liquid chromatography; OPBA, 4-oxo-4-(3-pyridyl)butyric acid; NNAL-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol; NNK-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

[4] L. A. Peterson, R. Mathew, and S. S. Hecht, unpublished results.

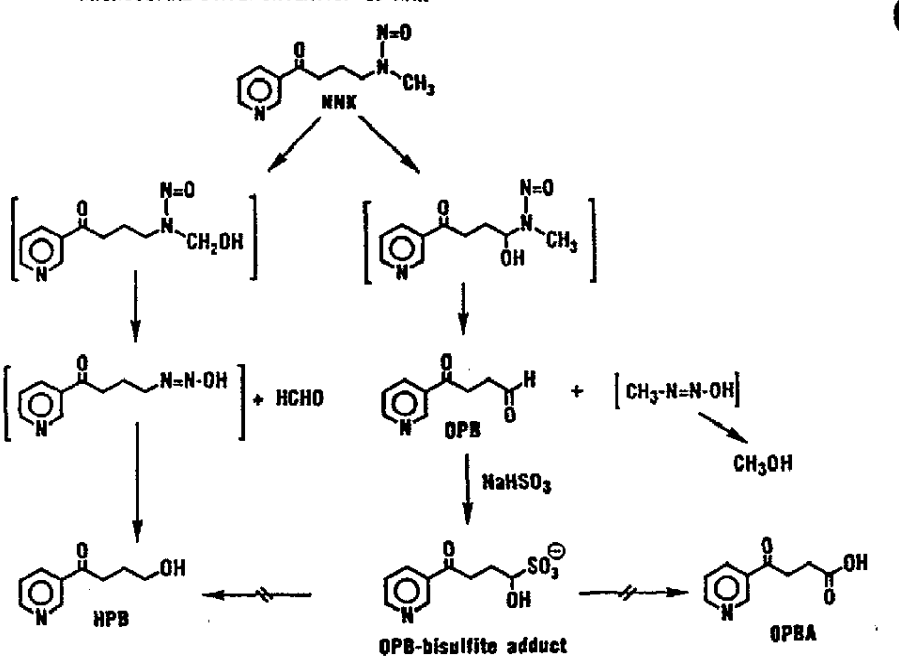


Fig. 1. α-Hydroxylation routes of NNK metabolism.

gradient was used: a linear gradient from 100% solvent C to 70% solvent C over 60 min followed by a 15-min gradient to 50% solvent C (flow: 1 ml/min). This system separates OPB-bisulfite adduct (14 min) and OPB (42 min), but other NNK metabolites coeluted.

Method C was developed to separate all the NNK metabolites and OPB-bisulfite adduct. This method uses solvent D (20 mM sodium phosphate buffer, pH 6, and 1 mM sodium bisulfite) and solvent B. Separation of the mixture was achieved by using a 60-min gradient from 100% D to 70% D, followed by a 10-min gradient to 50% D. The best separation of the metabolites was obtained by using a Phenomenex C_{18} column (Bondaclone, 10 μ, 300 x 3.9 mm) with the metabolites having the following retention times (min): OPB-bisulfite adduct, 20; OPBA, 22; NNAL-N-oxide, 28; 4-hydroxy-1-(3-pyridyl)-1-butanol, 32; NNK-N-oxide, 35; HPB, 44; NNAL, 49; and NNK, 58 min.

Picofluor (Packard Instrument Co., Meridian, CT) was the scintillation cocktail of choice in this chromatographic system since it gave low background radioactivity. High background was observed with Monofluor (National Diagnostic, Palmetto, FL), presumably a result of its interaction with the bisulfite in the mobile phase. It was important to prepare solvent D each day since the bisulfite air oxidized to sulfuric acid over a 24-h time period. This process could be retarded by sufficient degassing of the buffer.

Characterization of OPB-Bisulfite Adduct. OPB (2 mM) was reacted with 10 mM sodium bisulfite in 20 mM sodium phosphate buffer, pH 7, at room temperature. OPB-bisulfite adduct was purified by reverse-phase HPLC chromatography by using HPLC method B. The fractions containing the reaction product were combined, concentrated, and analyzed on the C_{18} column by using 90% H_2O and 10% methanol. The appropriate fractions were concentrated and the residue was resuspended in D_2O for nuclear magnetic resonance analysis, using a Bruker Model AM 360 WB spectrometer. See Table 2 for proton assignments.

Mass spectra were obtained on a Hewlett Packard Model 5988A spectrometer. The mass spectra of the OPB-bisulfite reaction product were identical to those of OPB (13), *m/e* (relative intensity): chemical ion mass spectrometry, M^+ 164 (100); electron impact mass spectrometry, 135 (36), 106 (100), and 78 (100).

Microsomal Incubations. Female A/J mice (6 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained on an AIN-76A semipurified diet (Dyets, Inc., Bethlehem, PA; No. 100,000 with 5% corn oil) for 1 week prior to sacrifice. Lung and liver microsomes were prepared from fresh tissue as previously described (14) and were stored at −80°C. Protein was quantitated by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA), with bovine serum albumin as a standard.

All incubations except where indicated were done in 100 mM phosphate buffer, pH 7, in the presence of 3 mM $MgCl_2$, 1 mM EDTA, 1 mM $NADP^+$, 5 mM glucose 6-phosphate, and 3.84 units/ml glucose-6-phosphate dehydrogenase. In all cases they were terminated by addition of 0.3 N barium hydroxide and 0.3 N zinc sulfate (0.25 ml each/ml incubation) prior to cooling on ice. The mixture was filtered through an Acrodisc (Gelman; 0.45 μM, 3 mm) and then analyzed directly by reverse-phase HPLC analysis with radiochemical detection (Flo-one/Beta, Radiomatics Instruments, Tampa, FL). Quantitation of the metabolites was achieved by measuring the radioactivity that coeluted with unlabeled standards.

Liver Microsomal Incubations. Initial studies involved duplicate incubations of [5-^{3}H]NNK (200 μM, 10 μCi/μmol) with liver microsomes (2 mg/ml; total volume, 1 ml) and cofactors in the presence or absence of 5 mM sodium bisulfite for 30 min at 37°C. The mixtures were analyzed directly by using HPLC method A.

The time course of NNK metabolism was determined by incubating 200 μM [5-^{3}H]NNK with liver microsomes (2 mg/ml) and cofactors in the presence of 5 mM bisulfite for 0, 5, 10, 15, 30, and 45 min. The duplicate samples were analyzed by using HPLC method C.

The dependence of NNK metabolism on protein concentration was examined by incubating 200 μM NNK in the presence of sodium bisulfite and cofactors with 0.1, 0.5, 1, 1.5, 2, 5, or 10 mg/ml liver microsomal protein. Triplicate incubations were conducted for 10 min at 37°C. HPLC method C was used for quantitation of the metabolites.

The apparent K_m and V_{max} for the NNK oxidation pathways were determined by incubating [5-^{3}H]NNK (1, 2.5, 5, 7.5, 10, 15, 20, 25, 50, or 100 μM; 1 μCi) with 0.25 mg/ml liver microsomal protein in the presence of 5 mM sodium bisulfite, 25 mM glucose 6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 5 mM $NADP^+$, 1 mM EDTA, and 3 mM $MgCl_2$ (total volume, 0.4 ml). The incubations were done in triplicate at 37°C for 10 min and were analyzed by HPLC method C. The data were analyzed by using simple Michaelis-Menten kinetics with a nonlinear curve-fitting program (15).

Lung Microsomal Incubations. Initial studies were performed by using 1.5 mg/ml lung microsomal protein and 200 μM [5-^{3}H]NNK (10 μCi/μmol) with cofactors (see above). The incubations were done in the presence or absence of 5 mM sodium bisulfite at 37°C for 15 or 30 min.

K_m and V_{max} studies were carried out with [5-^{3}H]NNK (0.5, 1, 2.5, 5, 10, 15, 20, 50, or 100 μM; 1 μCi), 5 mM sodium bisulfite, 25 mM glucose 6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 5 mM $NADP^+$, 1 mM EDTA, 3 mM $MgCl_2$, and 0.25 mg/ml lung microsomal protein (total volume, 0.4 ml) (8). The incubations were done in triplicate for 30 min at 37°C and were analyzed directly by using HPLC method C. The data were analyzed as described above.

Metabolism of HPB. [5-^{3}H]HPB (0.1, 0.2, or 1.0 μM; 125.5 μCi/μmol) was incubated with lung or liver microsomes (0.25 mg/ml) in the presence of cofactors and 5 mM sodium bisulfite as described in the K_m and V_{max} studies. The analyses of the triplicate incubation mixtures were performed by using HPLC method C.

Recovery of [5-^{3}H]OPB. [5-^{3}H]OPB was prepared by incubating [5-^{3}H]NNK (50 μmol, 250 or 500 μCi/μmol) with liver microsomes (2 mg protein/ml), cofactors, and 5 mM sodium bisulfite for 60 min at 37°C. The protein was precipitated with barium hydroxide and zinc sulfate. The [5-^{3}H]OPB-bisulfite adduct was isolated from the incubation mixture by using HPLC method B. In this system, the OPB-bisulfite adduct eluted at 14 min. The appropriate fraction was collected, the pH was adjusted to 7, and the methanol was removed. Under neutral conditions, the adduct is in equilibrium with free OPB. The adduct (14 min) and free OPB (42 min) were separated by analyzing the sample on the same HPLC system. The fractions containing OPB were collected and the methanol was removed. This fraction was applied to a C_{18} Sep-Pak (Waters, Melford, MA), and [5-^{3}H]OPB was eluted with methanol, concentrated, and stored at −20°C in methylene chloride. The identity and purity of OPB were confirmed by cochromatography with unlabeled OPB in HPLC methods A, B, and C.

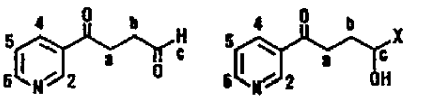
[5-^{3}H]OPB (0.01, 0.1, and 0.5 μM, 500 μCi/μmol) was incubated with 5 mM sodium bisulfite in the presence or absence of 0.25 mg/ml liver microsomal protein and cofactors for 10 min. The duplicate samples were analyzed by using HPLC method C.

RESULTS

In order to determine the relative rates of the two α-carbon oxidation routes of NNK, a reliable method was needed for the quantitation of the reactive ketoaldehyde, OPB. Incubations of [5-^{3}H]NNK with A/J mouse liver microsomes were conducted in the presence of several reagents that had potential for trapping OPB *in situ*. The reagents included lysine, semicarbazide, methoxyamine, and sodium bisulfite (data not shown). Only bisulfite gave a single OPB reaction product without inhibiting NNK metabolism.

Initially, liver microsomal incubations were analyzed on a C_{18} column by using 20 mM sodium phosphate buffer, pH 7, with a linear methanol gradient (HPLC method A; Ref. 12). In this system, OPB-bisulfite adduct coeluted with HPB. Therefore, incubations conducted in the presence of bisulfite contained higher apparent levels of HPB than incubations performed in the absence of bisulfite (Table 1). When bisulfite was included, OPBA levels were reduced but none of the other NNK metabolic pathways were affected.

Table 2 *^{1}H-Nuclear magnetic resonance chemical shift assignments for OPB, OPB hydrate, and the OPB-bisulfite adduct at neutral pH*[a]



Protons	OPB (ppm)	OPB hydrate X = OH (ppm)	OPB-bisulfite adduct X = SO_3^- (ppm)
2	9.1	9.1	9.1
4	7.6	7.6	7.6
5	8.35	8.35	8.35
6	8.7	8.7	8.7
a	3.5	3.2	3.4
b	3.0	2.0	2.1, 2.4
c	9.75	5.2	4.5

[a] Sodium 3-trimethylsilylpropanoate-d_4 in D_2O was used as an external reference.

Chemical characterization of the OPB-bisulfite reaction product demonstrated that bisulfite adds solely to the aldehyde moiety. The adduct was stable in the absence of excess bisulfite under acidic conditions (pH ~ 4) for more than 24 h as judged by HPLC analysis. The nuclear magnetic resonance spectrum of the adduct at neutral pH contained signals from three different chemical species, OPB (aldehyde proton at 9.75 ppm), OPB hydrate (5.1 ppm), and OPB-bisulfite adduct (4.5 ppm) (see Table 2). The pyridine proton signals for all three compounds were identical, indicating that the adduct retained the ketone carbonyl group. These results suggest that the adduct is stable at low pH but is in equilibrium with the free aldehyde (and its hydrate) at higher pH.

Electron impact and chemical ion mass spectra of OPB-bisulfite adduct were identical to those of OPB (13). The only difference between the two compounds was the temperature at which they volatilized; the bisulfite adduct required substantially higher temperatures. This observation demonstrates that the adduct is thermally unstable and decomposes to OPB in the mass spectrometer source. All these data indicate that formation of the OPB-bisulfite addition product is reversible. Therefore, to ensure adduct formation, NNK microsomal incubations were conducted with excess bisulfite.

In order to determine the relative extent of α-hydroxylation at the two carbon atoms, an HPLC system was required to separate OPB-bisulfite adduct from other NNK metabolites. This separation was achieved by using sodium phosphate buffer, pH 6, and 1 mM bisulfite with a linear methanol gradient (Fig. 2). If bisulfite was not included, OPB-bisulfite adduct gave a broad peak at 20 min with a smaller peak eluting with HPB.

Table 1 *Liver and lung microsomal metabolism of [5-^{3}H]NNK in the absence and presence of sodium bisulfite*

Source of microsomes	$NaHSO_3$	NNK metabolites (pmol/mg protein/min)						
		OPB	OPBA	NNAL-N-oxide	NNK-N-oxide	HPB	NNAL	NNK
Liver[a]	−	[b]	170	11.7	17.7	243[b]	527	2130
	+	[b]	35	11.7	15.0	370[b]	510	2190
Lung[c]	−	ND[d]	4.9 ± 0	ND	20.2 ± 0.4	21.1 ± 0.9	149 ± 2	4160 ± 0
	+	12.0 ± 0.2[e]	1.8 ± 0.7	ND	22.2 ± 0.2	16.7 ± 1.1	142 ± 9	4170 ± 10

[a] Incubations were done with 200 μM [5-^{3}H]NNK in the presence of A/J mouse liver microsomes (2 mg/ml), and an NADPH-generating system in the presence or absence of 5 mM $NaHSO_3$ for 30 min at 37 °C. Samples were analyzed by HPLC method A. Values are mean of two samples.

[b] The OPB-bisulfite adduct, OPB, and HPB coeluted under the conditions used to analyze the liver microsomal samples.

[c] Incubations were done as above except that the lung microsomal protein concentration was 1.5 mg/ml. These samples were analyzed by HPLC method C which allows detection of OPB-bisulfite adduct. Values are the mean of three samples ± SD.

[d] ND, not detected.

[e] OPB is detected as the OPB-bisulfite adduct.

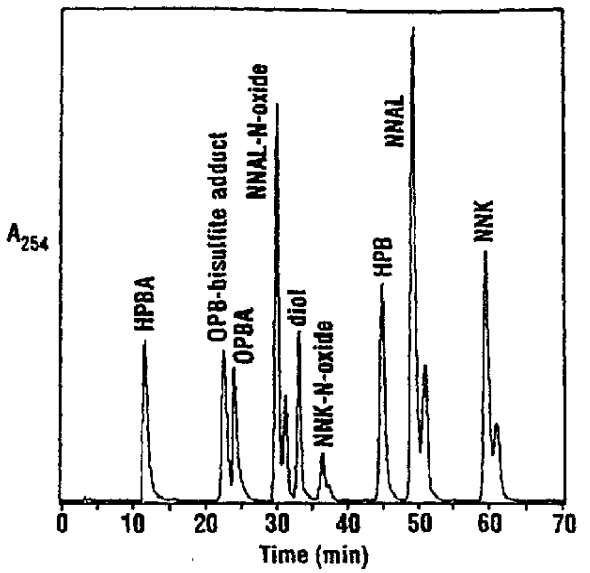


Fig. 2. Separation of NNK and its metabolites by using HPLC method C.

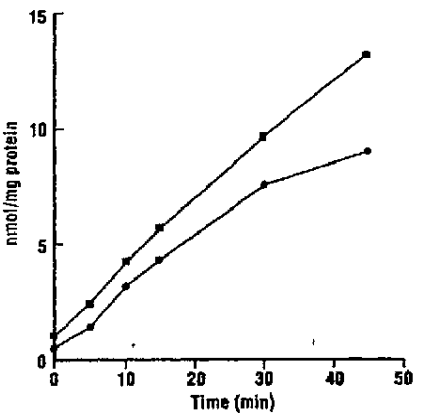


Fig. 3. Time course of NNK metabolism in mouse liver microsomes. Incubations were done with 200 μM [5-^{3}H]NNK in the presence of mouse liver microsomes (2 mg protein/ml), an NADPH-generating system, and 5 mM bisulfite for 0–45 min at 37°C. Values are the means of two samples (the individual samples were within 10% of the mean); OPB, ■; HPB, ●.

The presence of excess bisulfite stabilized the adduct without influencing the retention times of other NNK derivatives.

The HPLC assay was used to determine the extent of NNK metabolism to OPB and HPB in liver microsomal preparations. Initial rate conditions were established by measuring the time course of NNK metabolism as well as the dependence on protein concentration (Figs. 3 and 4, respectively). These studies established the following initial rate conditions: 10 min at 37°C in the presence of 0.25 mg microsomal protein/ml. The studies of the concentration dependence of liver microsomal oxidation of NNK were performed with a liver microsomal preparation that had been stored for 4 months at −80°C. The effects of storage on liver microsomal NNK metabolic activity have not been fully investigated. A representative radiogram is displayed in Fig. 5*A*. Liver microsomes were able to convert NNK to OPB, HPB, and NNAL. OPBA was not detected. The levels of OPB were greater than those of HPB (Fig. 6). The ratio of OPB to HPB was approximately 1.5. The apparent K_m for HPB formati (73.8 μM) was almost 4-fold higher than that for OPB formati (19.1 μM; Table 3).

As with liver microsomes, preliminary studies indicated th bisulfite did not inhibit the rate of NNK metabolism in lu microsomes (Table 1). Initial rate studies with lung microsom were conducted under conditions established by Smith *et* (8): 30 min at 37°C in the presence of 0.25 mg microsom protein/ml and essential cofactors. A representative radiogra is displayed in Fig. 5*B*. Like liver microsomes, lung microsom were capable of metabolizing NNK to OPB, HPB, and NNA NNK-N-oxide, as well as an unknown eluting at 26 min, we also detected. No measurable levels of OPBA were observed these incubations. The levels of OPB and HPB detected in the incubations are displayed in Fig. 6. The amount of HPB e ceeded that of OPB at NNK concentrations less than 15 μM These two metabolites were formed in comparable amounts higher NNK concentrations.

Thus, liver microsomes were substantially more ac i

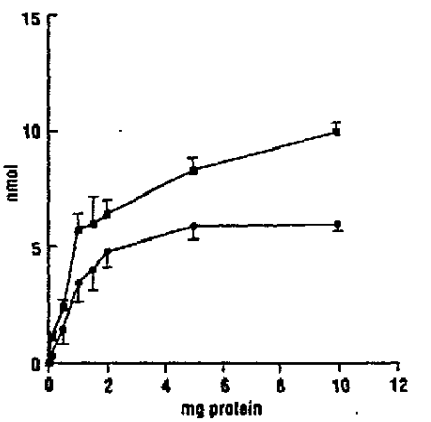


Fig. 4. Dependence of NNK metabolism on liver microsomal protein concentration. Incubations were done with 200 μM [5-^{3}H]NNK in the presence of mouse liver microsomes (0.1–10 mg protein/ml), an NADPH-generating system 5 mM bisulfite for 10 min at 37°C. *Points*, means of three determinations; O. ., ■; HPB, ●; *bars*, SD.

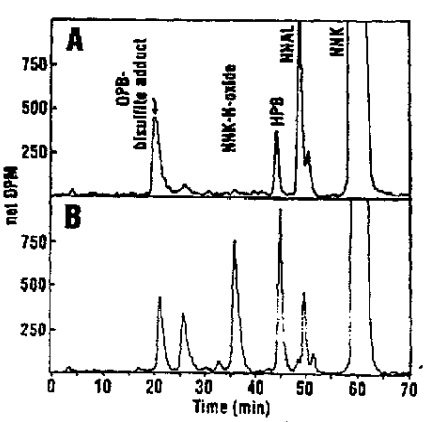


Fig. 5. Representative HPLC radiograms (HPLC method C) of NNK metabolites formed in (*A*) mouse liver microsomes and (*B*) mouse lung microsomes. The *in vitro* mixtures consisted of 10 μM [5-^{3}H]NNK, microsomal protein (0.25 mg/ml), and NADPH-generating system, and 5 mM $NaHSO_3$. The incubations were carried out at 37°C for 10 min with liver microsomes and for 30 min with lung microsomes.

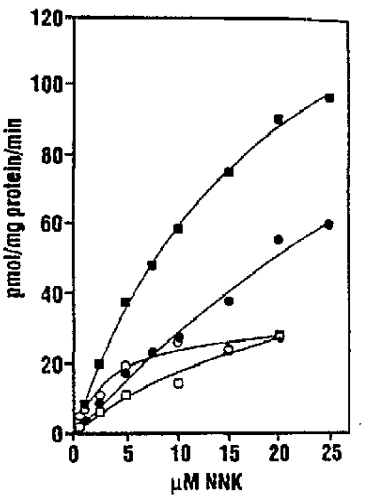


Fig. 6. Substrate dependency of NNK metabolism to OPB (■) and HPB (●) *in lung* (□, ○) and liver (■, ●) *microsomes.* The incubation mixtures consisted of [5-^{3}H]NNK (0.5–25 μM), microsomal protein (0.25 mg/ml), an NADPH-generating system, and 5 mM bisulfite. The incubations were conducted at 37°C for 10 min with liver microsomes and for 30 min with lung microsomes. These data were generated in a single experiment; *points*, mean of three samples.

converting NNK to OPB than were lung microsomes. The levels of HPB formed in incubations with less than 10 μM NNK were similar in lung and liver microsomes. However, liver preparations were more active than lung preparations at higher concentrations. This difference is reflected in the 20-fold difference in apparent K_m for HPB formation for lung microsomes (3.6 μM) *versus* liver microsomes (73.8 μM; Table 3). The apparent K_m for OPB formation was similar for both lung and liver preparations (23.7 and 19.1 μM; Table 3).

A possible confounding factor in these studies is the potential for further metabolism of HPB to OPB, or OPB to either HPB or OPBA. When [5-^{3}H]HPB (0.1, 0.2, and 1.0 μM) was incubated with liver microsomes, less than 15% of [5-^{3}H]HPB was metabolized to [5-^{3}H]OPB. No detectable oxidation of HPB (0.1 μM) to OPB was observed in lung microsomes. Between 98 and 100% of [5-^{3}H]OPB (0.01–0.5 μM) could be recovered as OPB under the conditions used for the initial rate studies with liver microsomes. The recovery of OPB was substantially reduced when higher protein concentrations were used (data not shown). This observation is consistent with the hypothesis that OPB can react with protein nucleophiles. Therefore, under the conditions of the initial rate studies, all of the OPB formed is detected and there is little or no interconversion between OPB and HPB.

DISCUSSION

An assay was developed to quantitate the primary metabolites of the two α-hydroxylation pathways of NNK bioactivation. HPB was used to measure the extent of methyl hydroxylation, whereas OPB was used to determine the extent of methylene hydroxylation. When NNK was incubated with lung or liver microsomes in the presence of bisulfite, the reactive aldehyde, OPB, was trapped *in situ* and was measured as the OPB-bisulfite adduct. Bisulfite has previously been shown to be a suitable trapping agent for aldophosphamide, an aldehyde metabolite of cyclophosphamide (16). The inclusion of bisulfite in microsomal incubations had no effect on the rate of NNK metabolism. The presence of bisulfite minimized the binding of OPB to microsomal proteins as well as the conversion of OPB to either HPB or OPBA. Under our reaction conditions, less than 15% of HPB was further oxidized to OPB. Therefore, this assay reliably determines the metabolism of NNK through the two activation pathways.

When this assay was used to determine rates of α-hydroxylation in A/J mouse liver and lung microsomes, measurable levels of both OPB and HPB were detected. However, there were some interesting tissue-specific differences in the relative rates of these two pathways. At concentrations greater than 10 μM, liver microsomes were more active in oxidizing NNK to both OPB and HPB than were lung microsomes. This observation is consistent with the levels of DNA methylation and pyridyloxobutylation detected in livers relative to lungs of NNK-treated mice (7, 17). The ratio of OPB to HPB formation was approximately 1.5 in liver preparations, whereas it ranged from 0.2 to 1 in lung microsomes. The apparent K_m for methylene hydroxylation (OPB formation) was similar for both lung and liver preparations, whereas there was a 20-fold difference in the apparent K_m for methyl hydroxylation (HPB formation). Together, these data are consistent with the involvement of multiple cytochrome P-450 isozymes in the two hydroxylation pathways.

A study by Smith *et al.* (8) provided evidence for the partial participation of P-450s IIB1 and 2 as well as P-450 IA1 in the methyl hydroxylation of NNK in mouse lung microsomes. Our apparent K_m for HPB formation (3.6 μM) is similar to what they reported (5.6 μM). They did not quantify the methylene hydroxylation route in their study. Devereux *et al.* (18) demonstrated that antibodies against rabbit P-450 IIB4 (orthologous to rat P-450b) inhibited DNA methylation by NNK in rat lung microsomes. Our assay will permit a more complete determination of the isozymes involved in the activation of NNK.

There is also evidence for the involvement of different isozymes for methyl *versus* methylene hydroxylation of NNK *in vivo*. The time courses of DNA methylation and pyridyloxobutylation are different from one another in A/J mouse lung following a single i.p. dose of 10 μmol NNK (6). DNA methylation peaked 4 h after NNK exposure, whereas DNA pyridyloxobutylation did not reach a maximum until 24 h after exposure. While these differences could be a result of a number of factors such as repair and reactivity of the alkylating agents, one likely explanation is that there are at least two different isozymes responsible for the two pathways. Our *in vitro* results support this hypothesis.

Methylation, as measured by O^6- and 7-methylguanine, was roughly 35 times greater than pyridyloxobutylation in A/J mouse lung following i.p. exposure to 10 μmol NNK (6). From these data, it appears that more methylating metabolites are formed than pyridyloxobutylating metabolites. However, we have observed that the methylating agent is about 2 orders of magnitude more reactive with calf thymus DNA *in vitro* than

Table 3 *Kinetic parameters for NNK metabolism in mouse liver and lung microsomes*[a]

	Metabolite	K_m (μM)	V_{max} (pmol/mg protein/min)	$\frac{V_{max}}{K_m}$
Liver	OPB	19.1 ± 1.2	173 ± 6	9.1
	HPB	73.8 ± 6.8	239 ± 11	3.2
Lung	OPB	23.7 ± 2.6	58.9 ± 2.6	2.5
	HPB	3.6 ± 0.9	32.5 ± 2.5	9.0

[a] Values are the mean ± SE of three replications.

is the pyridyloxobutylating agent.[4] Therefore, the *in vitro* observation that lung microsomes are equally or less active in generating the methylating agent than they are in producing pyridyloxobutylating metabolites is consistent with the *in vivo* data.

In summary, we have developed an assay that permits the determination of the levels of HPB and OPB formed during the *in vitro* metabolism of NNK. This system provides a simple and straightforward means to study the biochemical parameters of the two α-hydroxylation pathways of NNK. It will be a useful tool in the study of NNK carcinogenesis.

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